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13. ABSTRACT ***(Maximum 200 Words)***

Dihydroxyvitamin D3 (VD3) affects essential cell processes such as proliferation, differentiation, and apoptosis in a diverse cell types, including cancer cells. In this study, we investigate whether VD3 could inhibit proliferation of breast cancer cells (BCCs) by suppressing the expression and signaling of endothelial differentiation gene-encoded G protein-coupled receptors (EDG Rs). EDG Rs transduce major effects of lysophospholipid growth factors (LGFs), such as lysophosphatidic acid (LPA) and sphingosine-1 phosphate (S1P), on estrogen receptor-negative and estrogen receptor-positive BCCs, including stimulation of proliferation and growth-independent functions. MDA-MB-453 and MCF-7 BCCs used in this study express predominantly Edg-3> 4 > 5 and –2, but not Edg-1. We have shown that VD3, at 10^{-10} to 10^{-8} M, suppressed significantly mRNA levels of Edg-2, -3, -5, but not Edg-4 in both lines of BCCs as quantified by TaqMan real-time PCR. VD3 also inhibited proliferative responses of LGF-stimulated BCCs and modulated LPA- and S1P- induced SRE reporter responses of BCCs. These data identify a novel mechanism of VD3 action on the abnormal growth of BCCs and suggest that EDG Rs are potential targets for specific therapy of some human breast cancers.

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5) INTRODUCTION

The active metabolite of vitamin D, 1α,25- dihydroxyvitamin D_3 (VD3) has been known to effect essential cell regulatory processes such as proliferation, differentiation, and apoptosis in a number of different cell types, including cancer cells. As the mechanism by which VD3 inhibits breast cancer cell (BCC) growth is not fully known yet, we investigated whether the effects of VD3 include inhibition of lysophospholipid growth factor (LGF)- stimulated proliferation of BCCs by suppressing endothelial differentiation gene-encoded G protein-coupled receptors (EDG Rs) expression and signaling.
EDG Rs transduce major effects of the LGFs, lysophosphatidic acid (LPA) and sphingosine-1 phosphate (S1P). EDG-1,-3,-5, -6 and –8 are specific for S1P and EDG-2,-4, and -7 are specific for LPA. LPA and S1P potently stimulate proliferation, enhance survival and inhibit apoptosis of many different normal and malignant cells.

6) BODY

a) As we reported in the previous annual summary the first task of the revised research plan was to examine the Edg Rs and responses to LPA and S1P of the MCF-7 and MDA-MB-453 BCCs. We showed that both cell lines express Edg-3> 4 > 5 and –2, but not Edg-1. The proliferation of both cell lines was significantly stimulated by 10-8-10-6M LPA and S1P as assessed by cell counts and quantification of stimulation of serum response element (SRE) transcription activity with Renilla luciferase-corrected reports from an SRE-luciferase plasmid transfected into the BCCs. The increase of secretion of radioimmunoreactive IGF-II by LGF-stimulated BCCs suggested that both estrogen receptor positive and -negative cell lines respond to LPA and S1P also by indirect, IGF-II- mediated mechanism.

b) Effects of VD3 on BCC Edg R Expression as Assessed by TaqMan Real Time Polymerase Chain Reaction (PCR) Technique.

In order to examine the Edg R expression more accurately we have adapted a TaqMan Real Time PCR technique in our model system. New sets of Edg R specific primers and fluorogenic probes were designed and real-time PCR analysis was performed using an Applied Biosystems Prism

model 7700 sequence detection instrument. The expression of Edg Rs was quantified using a comparative cycle threshold method for quantifying target DNA sequences as previously reported (1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ß-glucuronidase (Gus) genes were used as the control "housekeeping" genes for the relative quantitation of the Edg R expression.
Real Time PCR technique confirmed our previous results obtained from semi-quantitative radioactive RT-PCR on MDA-MB-453 and MCF-7. Both BCCs expressed EDG-3 > EDG-4> EDG-5> EDG-2 Rs without detectable EDG-1.

The following experiments were focused on the real -time PCR quantification of Edg R expression in VD3 – treated BCCs. The cells were treated with VD3, at 10-9-10-7 M, for 4, 8, 24 and 48 hour intervals to determine maximally-inhibitory concentrations and exposure times of VD3 on Edg R expression by breast cancer cells.

Conclusion: VD3 suppressed significantly the levels of mRNA encoding EDG-2 (mean=65%), -3 (mean =50%), and –5 (mean=40%), but didn't effect expression of EDG-4 in MDA-MB-453 BCCs (Table 1a).
VD3 affected the expression of EDG-2, -3 and –5 in MCF-7 BCCs to the same extend as in MDA-MB-453 BCCs, with the exception of EDG-4, which expression was also suppressed at the earlier time point (mean=20%) (Table 1b).

c) VD3 Modulation of LGF-Induced SRE-Reporter Response of Transiently Transfected Breast Cancer Cells.

VD3 inhibition of proliferative responses of LGF-stimulated BCCs was assessed with quantification of stimulation of serum response element (SRE) transcription activity with Renilla luciferase-corrected reports from an SRE-luciferase plasmid transfected into the BCCs.

Conclusion: VD3, at 10-8M, suppressed the response of MDA-MB-453 cells by a mean of 91%. However, VD3 hasn't significantly affected the SRE- response of MCF-7 BCCs.

d) VD3 Modulation of [Ca++]i Evoked by LPA and S1P in BCCs.

In this study we sought to determine the effect of 1α,25- dihydroxyvitamin D_3 on Edg R mediated signaling. VD3 effect on Edg R- mediated intracellular calcium mobilization induced by S1P and/or was assessed fluorometrically with Fura-2.
Conclusion: Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) induced an increase in intracellular ([Ca2+]i in MDA-MB-453 BCCs in dose dependent manner (3000 fold).

The effect of VD3 on S1P– induced Ca(2+) mobilization in MDA-MB-453 BCCs was similar to that of U-73122, a blocker of phospholipase C (PLC) (mean=45%). However, VD3 suppressed LPA-stimulated Ca(2+) mobilization in MDA-MB-453 BCCs to much lesser extend (mean=5%). Combination of VD3 and U-73122 treatment led to a significant decrease in [Ca2+](i) only in S1P-stimulated MDA-MB-453 cells (mean=86%).

7) List of Key Research Accomplishments:

Development of the new sets of Edg R specific primers and fluorogenic probes designed for TaqMan Real -Time PCR technique.

Characterization of the profile of Edg Rs for LPA and S1P expressed by MCF-7 (estrogen receptor positive) and MDA-MB-453 (estrogen receptor negative) human breast cancer cell lines by Real Time PCR technique.

Finding that dihydroxyvitamin D3 suppresses the expression of Edg-2, -3, and -5 in MDA-MB-453 and MCF-7 BCCs as assessed by Real Time PCR technique.

Demonstration of VD3- mediated suppression of LGF-induced SRE-luciferase reports from MDA-MB-453 BCCs.

Finding that VD3 suppresses S1P-induced intracellular calcium mobilization in MDA-MB-453 BCCs.

8) Reportable Outcomes

Full publications:

Goetzl, E.J., **Dolezalova, H.**, Kong, Y., and Zeng, L. Dual mechanism for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res., *59:* 4732-4737, 1999.

Goetzl, E.J., Lee, H., **Dolezalova, H.**, Kalli, K.R., Conover, C.A., Hu, Y.L., Azuma, T., Stossel, T.P., Karliner, J.S., Jaffe, R.B. Mechanisms of lysolipid phosphate effects on cellular survival and proliferation. Annals of the New York Academy of Sciences, 905:177-87, 2000.

Abstracts:

Goetzl, E.J., **Dolezalova, H.**, Kong, Y., and Zeng, L. Dual mechanism for endothelial differentiation gene-encoded receptor (Edg R) mediation of proliferation of human breast cancer cells. FASEB J., 13: A136

Dolezalova, H., Bikle,D.D. and Goetzl, E.J. Effect of 1,25- dihydroxyvitamin D3 on breast cancer cell growth. Proceedings, Vol. I, p. 292. Era of Hope DOD Breast Cancer Res. Program Meeting, Atlanta, 2000.

Dolezalova, H. and Goetzl, E.J. Suppression by 1,25-dihydroxyvitamin D3 of expression of endothelial differentiation gene - encoded G protein - coupled receptors (EDG Rs) for lysophospholipid growth factors. AACR 92nd Annual Meeting, New Orleans, 2001 in press.

- Full paper in preparation.

9) References

Anonymous. User Bulletin #2. ABI PRISM 7700 Sequence Detection System. Foster City, CA: PE Applied Biosystems. 1997.

10) Appendices

1)

Time of VD3 treatment (h)	Edg-2	Edg-3	Edg-4	Edg-5
8	65	50	0	20
24	60	40	0	40
48	0	43	0	35

Table 1a) Inhibition of Expression of Edg-2 – 5 in 1,25-Dihydroxyvitamin D3-treated MDA-MB-453. Inhibition of Edg R expression is shown as the mean percentage compared to an endogenous reference (VD3-untreated control =100%).

Time of VD3 treatment (h)	Edg-2	Edg-3	Edg-4	Edg-5
8	60	40	20	40
24	60	40	10	20
48	64	40	0	0

Table 1b) Inhibition of Expression of Edg-2 – 5 in 1,25-Dihydroxyvitamin D3-treated MCF-7. Inhibition of Edg R expression is shown as the mean percentage compared to an endogenous reference (VD3-untreated control =100%).

[CANCER RESEARCH 59, 4732–4737, September 15, 1999]

Dual Mechanisms for Lysophospholipid Induction of Proliferation of Human Breast Carcinoma Cells[1]

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ABSTRACT

Endothelial differentiation gene-encoded G protein-coupled receptors (Edg Rs) Edg-1, Edg-3, and Edg-5 bind sphingosine 1-phosphate (S1P), and Edg-2 and Edg-4 Rs bind lysophosphatidic acid (LPA). LPA and S1P initiate ras- and rho-dependent signaling of cellular growth. Cultured lines of human breast cancer cells (BCCs) express Edg-3 > Edg-4 > Edg-5 > or = Edg-2, without detectable Edg-1, by both assessment of mRNA and Western blots with rabbit and monoclonal mouse anti-Edg R antibodies. BCC proliferation was stimulated significantly by 10^{-9} M to 10^{-6} M LPA and S1P. Luciferase constructs containing the serum response element (SRE) of growth-related gene promoters reported mean activation of BCCs by LPA and S1P of up to 85-fold. LPA and S1P stimulated BCC secretion of type II insulin-like growth factor (IGF-II) by 2–7-fold, to levels at which exogenous IGF-II stimulated increased proliferation and SRE activation of BCCs. All BCC responses to LPA and S1P were suppressed similarly by pertussis toxin, mitogen-activated protein kinase kinase inhibitors, and C3 exoenzyme inactivation of rho, suggesting mediation by Edg Rs. Monoclonal anti-IGF-II and anti-IGFR1 antibodies suppressed proliferation and SRE reports of BCCs to LPA and S1P by means of up to 65%. Edg Rs thus transduce LPA and S1P enhancement of BCC growth, both directly through SRE and indirectly by enhancing the contribution of IGF-II.

INTRODUCTION

The lysolipid phosphate mediators LPA[3] and S1P are generated enzymatically from membrane lipid precursors of many different types of normal and malignant cells (1, 2). Extracellular LPA and S1P both stimulate cellular proliferation, differentiation, survival, adhesion, aggregation, and other specific functions (3–5). A recently characterized subfamily of at least five G protein-coupled receptors, which are encoded by *edg*s, bind and transduce signals from LPA or S1P (6–10). Two homology clusters with greater structural similarity and shared ligand specificity are composed of the *edg*-encoded G protein-coupled receptors (Edg Rs) Edg-1, Edg-3, and Edg-5 set of S1P Rs and Edg-2 and Edg-4 LPA Rs. The capacity of LPA and S1P to improve cellular survival is in part a result of suppression of apoptosis by several distinct mechanisms (11, 12). LPA and S1P stimulate cellular proliferation directly by eliciting the serum response factor and ternary complex factor transcription factors, which together bind to and activate the SRE in promoters of many immediate-early genes (13). The involvement of SRE-dependent mechanisms in mediating LPA and S1P enhancement of proliferation has not been examined carefully in malignant cells, nor has the possibility of effects of LPA and/or S1P on polypeptide growth factors necessary for optimal tumor growth.

Functional Edg receptors and proliferative responses to LPA and S1P thus were characterized in the ER-positive MCF-7 cultured line of human BCCs and the MDA-MB-453 ER-negative line of BCCs. The relative contributions of direct SRE-dependent induction of transcription and of enhancement of production of IGF-II in proliferative responses to LPA and S1P also were determined in these BCCs.

MATERIALS AND METHODS

Chemical Reagents and Antibodies. The sources of chemicals were: S1P and sphingosine (Biomol, Plymouth Meeting, PA); LPA, phosphatidic acid, 1-β-D-galactosyl-sphingosine (psychosine), and fatty acid-free BSA (Sigma Chemical Co., St. Louis, MO); and human IGF-II (Peprotech, Inc., Rocky Hill, NJ). Cells were treated with PTX (Calbiochem, Inc., La Jolla, CA), recombinant *Clostridium botulinum* C3 ADP-ribotransferase (C3 exoenzyme; List Biological Laboratories, Inc., Campbell, CA), which ADP-ribosylates rho specifically, and the MEK inhibitor 2′-amino-3′-methoxyflavone (PD98059; Calbiochem) as described (10, 14). Mouse monoclonal antibodies specific for substituent peptides of human Edg-3 (amino acids 1–21), Edg-4 (amino acids 9–27), and Edg-5 (amino acids 303–322) have been described (12, 15), the immunogens for which were selected from sequences of high homology among humans and rodents. The expected cross-reaction with corresponding rodent Edg Rs has been confirmed by the identical recognition of human and rat Edg-5 Rs. The cross-reactivity of each antibody with heterologous Edg proteins was <1%, as determined by Western blots of 0.1–100 μg of membrane proteins isolated from HTC4 rat hepatoma cells stably transfected with human Edg-2, Edg-3, Edg-4, or Edg-5 (12, 15). Each monoclonal IgG was purified by protein A affinity-chromatography (Pierce Chemical Co.) and used to develop Western blots at 0.1–0.3 μg/ml (15). A mouse monoclonal IgG1 that specifically neutralizes activity of human/rat IGF-II, but not IGF-I (Upstate Biotechnology, Inc., Lake Placid, NY), and a mouse monoclonal antibody, termed α-IR3, which blocks binding of IGF-II to IGFR1 (Oncogene Science, Cambridge, MA), were purchased. A rabbit polyclonal antiserum to rodent and human Edg-2 was kindly provided by Dr. Jerold Chun (University of California–San Diego, San Diego, CA).

Cell Culture and Quantification of Cellular Proliferation. Layers of ER-positive MCF-7 (ATCC # HTB-22) and ER-negative MDA-MB-453 (ATCC# HTB-131) human BCCs were cultured in DMEM with 4.5 g/100 ml of glucose, 10% FBS, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin (complete DMEM) to 100% confluence and relayered every 3–4 days to 25–30% confluence. To assess proliferation, replicate layers of 1×10^4 BCCs were cultured in 48-well plates in complete DMEM for 4 h, washed once, and cultured for 20 h in serum-free DMEM. Some wells were pretreated with PTX for 6 h, C3 exoenzyme for 30 h, or MEK inhibitor for 2 h. Antisera were added, followed in 1 h by lipid stimuli and incubation for 72 h. Then wells were washed two times with Ca^{2+}- and Mg^{2+}-free Hanks' solution, and the cells were harvested in 0.2 ml of EDTA-trypsin solution for staining with trypan blue and eosin and quantification by microscopic counting of 10 1-mm^3 fields in a hemocytometer.

Reverse Transcription-PCR Analysis of Edg Rs. Total cellular RNA was extracted by the TRIzol method (Life Technologies, Inc., Grand Island, NY), from suspensions of BCCs and lines of stably transfected rat HTC4 hepatoma cells, that all had low background expression of native Edg Rs, and each overexpressed one recombinant human Edg R. A Superscript kit (Life Technologies, Inc.) was used for reverse transcription synthesis of cDNAs. PCR began with a "hot start" at 94°C for 3 min; Taq DNA polymerase was added, and amplification was carried out with 35 cycles of 30 s at 94°C, 2 min at 55°C, and 1 min at 72°C. Two μCi of [α-^{32}P]dCTP were added to some sets

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[3] The abbreviations used are: LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; *Edg*, endothelial differentiation gene; SRE, serum response element; ER, estrogen receptor; BCC, breast cancer cell; MEK, mitogen-activated protein kinase kinase; PTX, pertussis toxin; IGF-II, type II insulin-like growth factor; IGFR, IGF receptor; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; G3PDH, glyceraldehyde 3-phosphate dehydrogenase.

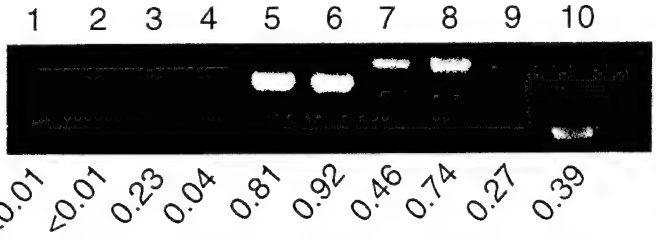

Fig. 1. RT-PCR semiquantification of mRNA encoding Edg Rs in MCF-7 and MDA-MB-453 cells. The volume of cDNA mixture from each type of BCC was selected to equalize the level of amplified G3PDH cDNA product. *Lanes 1, 3, 5, 7,* and *9* are from MDA-MB-453 cells, and *Lanes 2, 4, 6, 8,* and *10* are from MCF-7 cells. *Lanes 1* and *2*, Edg-1; *Lanes 3* and *4*, Edg-2; *Lanes 5* and *6*, Edg-3; *Lanes 7* and *8*, Edg-4; *Lanes 9* and *10*, Edg-5. The number *below* each lane represents the ratio of ^{32}P in cDNA for an Edg R to that for G3PDH.

of reaction mixtures to allow quantification of mRNA encoding each Edg R relative to that of the standard G3PDH (16). Oligonucleotide primer pairs were: G3PDH, 5′-dCCTGGCCAAGGTCATCCATGACAAC and 5′-dTGTCATACCAGGAAATGAGCTTGAC; Edg-1, 5′-CTACACAAAAAGCTTGGATCACTCA and 5′-CGACCAAGTCTAGAGCGCTTCCGGT (1100 bp); Edg-2, 5′-dGCTCCACACACGGATGAGCAACC and 5′-GTGGTCATTGCTGTGAACTCCAGC (621 bp); Edg-3, 5′-dCAAAATGAGGCCTTACGACGCCA and 5′-dTCCCATTCTGAAGTGCTGCGTTC (701 bp); Edg-4, 5′-dAGCTGCACAGCCGCCTGCCCCGT and 5′-dTGCTGTGCCATGCCAGACCTTGTC (775 bp); and Edg-5, 5′-CTCTCTACGCCAAGCATTATGTGCT and 5′-ATCTAGACCCTCAGACCACCGTGTTGCCCTC (500 bp). PCR products were resolved by electrophoresis in a 2 g/100 ml agarose gel with ethidium bromide staining. G3PDH and Edg R cDNA bands were cut from gels and solubilized for β-scintillation counting in 0.5 ml of sodium perchlorate solution at 55°C for 1 h (Elu-Quick; Schleicher and Schuell, Keene, NH). Initially, the G3PDH cDNA templates in several different-sized portions of each sample were amplified to determine volumes that would result in G3PDH bands of equal intensity for each sample. Relative quantities of cDNA encoding each Edg R also were calculated by the ratio of radioactivity to that in the corresponding G3PDH band (16).

Western Blots. Replicate suspensions of 1×10^7 BCCs, which had been incubated without or with LPA or S1P for 16 h, were washed three times with 10 ml of cold Ca^{2+}- and Mg^{2+}-free PBS, resuspended in 0.3 ml of cold 10 mM Tris-HCl (pH 7.4) containing a protease inhibitor mixture (Sigma Chemical Co., St. Louis, MO), 0.12 M sucrose, and 5% glycerol (v/v). After homogenization with a Teflon pestle on ice for 2 min at 250 rpm, each sample was centrifuged at $400 \times g$ for 5 min at 4°C, and the supernatant was centrifuged at $300,000 \times g$ for 30 min at 4°C. Each $300,000 \times g$ pellet was resuspended in 0.2 ml of 10 mM Tris-HCl (pH 7.4) with 1% (v/v) NP40, 5% glycerol, and protease inhibitor mixture and rehomogenized and incubated at 4°C for 2 h prior to centrifugation again at $300,000 \times g$. Aliquots of supernatant containing 1–100 μg of protein were mixed with 4× Laemmli's solution, heated to 100°C for 3 min, and electrophoresed in an SDS-12% polyacrylamide gel for 20 min at 100 V and 1.5 h at 140 V, along with a rainbow prestained set of molecular weight markers (DuPont NEN, Boston, MA or Amersham, Inc., Arlington Heights, IL). Proteins in each gel were transferred electrophoretically to a nitrocellulose membrane (Hybond; Amersham) for sequential incubation with 5 g% reconstituted nonfat milk powder to block unspecific sites, dilutions of mouse monoclonal anti-Edg R antibody, and then horseradish peroxidase-labeled goat anti-mouse IgG, prior to development with a standard ECL kit (Amersham).

RIA and Dot-Blot Quantification of IGF-II. RIAs were conducted according to the instructions of Research and Diagnostic Antibodies, Inc. (Berkeley, CA), after removal of some IGF binding proteins by Sep-Pak chromatography (Millipore Corp., Milford, MA), as directed (17). Dot-blot quantification of IGF-II was performed using a method in which binding proteins do not alter immunoreactivity of IGF-II in unprocessed cellular secretions (18).

Transfections and Reporter Assay. Replicate suspensions of $0.3–1 \times 10^5$ MCF-7 and MDA-MB-453 BCCs in 1 ml of complete DMEM were cultured in 12-well plates for 24 h to establish monolayers of 40–50% confluency. The monolayers were washed twice and covered with 1 ml of serum-free DMEM and lipotransfected with 100 ng/well of a SRE firefly luciferase reporter plasmid (8) and 5 ng/well of pRL-CMV Renilla luciferase vector (Promega Corp., Madison, WI) using FuGENE 6 (Boehringer Mannheim Corp., Indianapolis, IN). After 30 h of incubation, medium was replaced with fresh serum-free DMEM and anti-IGFR1 or anti-IGF-II mouse monoclonal antibodies or IgG1 isotype control was added, followed in 2 h by 10^{-10} M to 10^{-6} M LPA, S1P, or other lipids in serum-free DMEM with 0.1 mg/ml of fatty acid-free BSA. Some wells were pretreated with PTX for 6 h, C3 exoenzyme for 30 h, or MEK inhibitor for 2 h. After 4 h of incubation at 37°C, the luciferases were extracted in Reporter lysis buffer (Promega), and their activities were quantified sequentially by luminometry using Luciferase Assay and Stop & Glo reagents (Promega), with integration of light emitted during the 15 s after addition of each reagent (EG & G Berthold microplate luminometer, model LB96V). Firefly luciferase values were corrected for differences in apparent transfection efficiency by expression as a ratio with Renilla luciferase signals in the corresponding samples.

RESULTS

BCC Expression of Edg Receptors. mRNA encoding individual Edg Rs had been detected by Northern blotting in some human tumor cells (7–9). The relative levels of mRNA encoding each of the Edg Rs in BCCs now have been semiquantified by RT-PCR (Fig. 1). Several

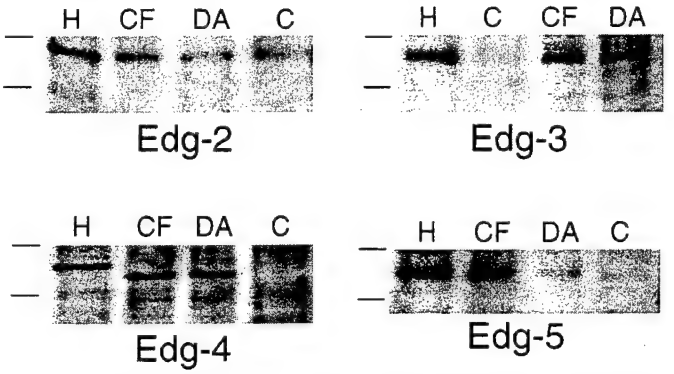

Fig. 2. Western blot analysis of the expression of Edg-2, Edg-3, Edg-4, and Edg-5 Rs by MCF-7 and MDA-MB-453 BCCs. The four samples analyzed for content of each Edg R are: *H*, 3 μg of protein extracted from HTC4 rat liver cells that were stably transfected with the respective Edg Rs; *C*, 10 μg of protein from control untransfected HTC4 cells; *CF*, 10 μg of protein from MCF-7 BCCs; and *DA*, 10 μg of protein from MDA-MB-453 BCCs. Blots were developed with rabbit anti-Edg-2 antiserum and anti-Edg-3, anti-Edg-4, and anti-Edg-5 mouse monoclonal antibodies. The marginal lines show the positions of M_r 45,000 and M_r 66,000 protein molecular weight markers.

Table 1 *Relative levels of mRNA encoding Edg receptors in BCCs*

All numbers are the mean ± SD of the results of three RT-radioPCR determinations of mRNA, where each value presented is the ratio of ^{32}P in the respective Edg R cDNA band to that in the G3PDH cDNA.

Type of BCC	Edg-1	Edg-2	Edg-3	Edg-4	Edg-5
MCF-7	0.00	0.04 ± 0.02	1.00 ± 0.08	0.70 ± 0.04	0.49 ± 0.09
MDA-MB-453	0.00	0.17 ± 0.05	0.81 ± 0.08	0.43 ± 0.03	0.19 ± 0.07
Edg-2 Tr[a]	<0.02	1.44 ± 0.17	0.18 ± 0.06	0.08 ± 0.06	0.06 ± 0.03
Edg-3 Tr	0.02	0.27 ± 0.09	1.29 ± 0.19	0.07 ± 0.05	0.03 ± 0.03
Edg-4 Tr	0.02	0.28 ± 0.03	0.16 ± 0.04	1.43 ± 0.28	0.07 ± 0.05
Edg-5 Tr	0.03	0.25 ± 0.07	0.17 ± 0.05	0.05 ± 0.04	1.36 ± 0.25

[a] Tr, HTC4 rat transfected hepatoma cells expressing a human recombinant Edg R.

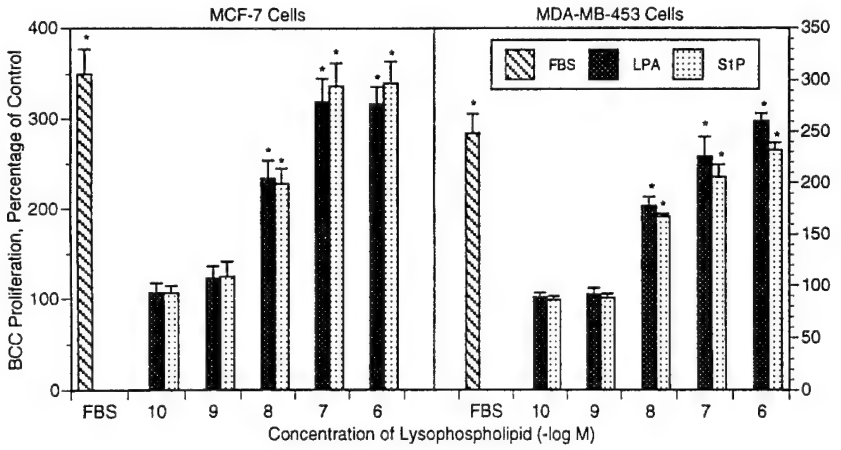

Fig. 3. Stimulation of proliferation of MCF-7 and MDA-MB-453 BCCs by LPA and S1P. *Columns*, means of the results of three studies performed in duplicate; *bars*, SD. FBS is the 2% FBS-positive control. The serum-free medium alone controls (100%) were 1.5, 1.4, and 1.5 × 10^4/well in the three studies of MCF-7 BCC proliferation and 1.1, 1.2, and 1.1 × 10^4/well in the three studies of MDA-MB-453 BCC proliferation. The levels of significance of increases above medium control proliferation were determined by a paired Student *t* test; *, $P < 0.01$.

different amounts of first-strand cDNAs prepared from MCF-7 and MDA-MB-453 BCCs were amplified initially to allow selection of a volume of each that provided equally intense cDNA bands for the internal standard G3PDH. With this standard approach, the mRNA from both human BCC lines was found to encode similarly high levels of Edg-3 R but had no detectable Edg-1 R message (Fig. 1). The ER-negative MDA-MB-453 BCCs had higher levels of mRNA encoding the Edg-2 R, whereas the ER-positive MCF-7 BCCs had higher levels of mRNA for Edg-4 and Edg-5.

RadioPCR has been used to assess levels of mRNA specific for other G protein-coupled Rs, but not Edg Rs (16). Thus, an initial study examined mRNA from four lines of rat HTC4 hepatoma cells, which were stably transfected with individual human Edg Rs 2 to 5 (Table 1). The rank order of levels of mRNA for endogenous Edg Rs in HTC4 cells prior to transfection was Edg-2 ≫ Edg-3 > Edg-4 > Edg-5, without detectable Edg-1 mRNA. The level of mRNA for the transfected Edg R in each line was much higher than background (Table 1). In this frame of reference, the levels of BCC mRNA encoding Edg-3 were nearly as high as the index transfectant and > Edg-4 > Edg-5 ≥ Edg-2, without any Edg-1 mRNA. The differences in relative amounts of mRNA for each Edg R between the two lines of BCCs were the same as for standard PCR (Fig. 1).

Western blots developed with polyclonal anti-Edg-2 R and monoclonal anti-Edg-3, anti-Edg-4, and anti-Edg-5 antibodies showed one predominant protein of expected size in extracts of each of the four lines of HTC4 cell transfectants (Fig. 2). Electrophoresis of over three times more protein from untransfected control HTC4 cells than transfectants did not show Edg-3, Edg-4, or Edg-5 protein antigen, but a faint band of Edg-2 protein was detected that might reflect the higher endogenous levels of mRNA encoding this R (Table 1). The results of BCC Western blots confirmed expression of Edg proteins representing both LPA R and S1P R subtypes, with a predominance of Edg-3 R in both BCC lines (Fig. 2). In contrast to expectations from PCR results, however, MCF-7 BCCs had higher levels of Edg-2 as well as Edg-4 and Edg-5 proteins than MDA-MB-453 BCCs. The Edg-4 R protein of both BCC lines was consistently M_r 2000–3000 smaller than the recombinant human Edg-4 R protein, but the basis for the difference has not yet been elucidated.

Functional and Biochemical Responses of BCCs to LPA and S1P. The proliferation of both lines of BCCs was assessed by counting viable cells after 72 h (Fig. 3). Proliferation of MCF-7 BCCs was increased significantly by 10^{-8} M to 10^{-6} M LPA and S1P to maximum levels similar to those attained by 2% FBS. In parallel studies of MDA-MB-453 BCCs, proliferative responses to LPA and S1P were similar to those of MCF-7 BCCs, with significant increases evoked by 10^{-8} M to 10^{-6} M LPA and S1P (Fig. 3).

Activation of SRE in the promoters of diverse growth-related genes is a fundamental characteristic of the growth-promoting potential of LPA and S1P. BCCs thus were transfected with an SRE-firefly luciferase construct and 1/20 the amount of a Renilla luciferase-CMV construct as an internal standard for consistency of transfection. LPA and S1P increased the mean levels of standardized luciferase luminometric activity in ligand concentration-dependent relationships by maxima of up to 37-fold and 85-fold, respectively, in MCF-7 BCCs (Fig. 4). Similar responses to the same concentrations of LPA and S1P

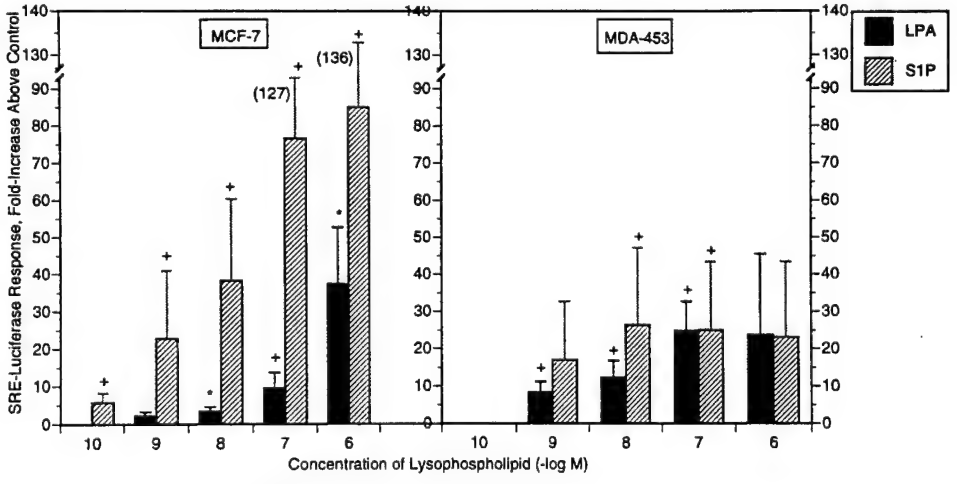

Fig. 4. SRE reporter assay of LPA and S1P stimulation of human BCCs. *Columns*, means of the results of three studies performed in duplicate; *bars*, SD. The medium alone control values were 1272, 957, and 352 luminometer units for MCF-7 BCCs and 269, 715, and 1401 for MDA-MB-453 BCCs. The statistical methods and symbols are the same as in Fig. 3, except that + = $P < 0.05$.

Table 2 *Pharmacological inhibition of LPA and S1P signaling to the SRE-Luciferase reporter in BCCs*

Each number is the mean of results of two studies performed in duplicate and presented as the percentage of inhibition of the control responses to 10^{-7} M LPA and 10^{-7} M S1P in serum-free DMEM without inhibitors (0% inhibition). Inhibitor conditions were 50 ng/ml of PTX for 6 h, 5 μM MEK inhibitor (MEK INH) for 1 h, and 10 μg/ml of C3 exoenzyme for 30 h.

	MCF-7 BCCs			MDA-MB-453 BCCs		
	PTX	MEK INH	C3 exoenzyme	PTX	MEK INH	C3 exoenzyme
LPA	74	41	41	80	69	75
S1P	60	37	44	78	61	79

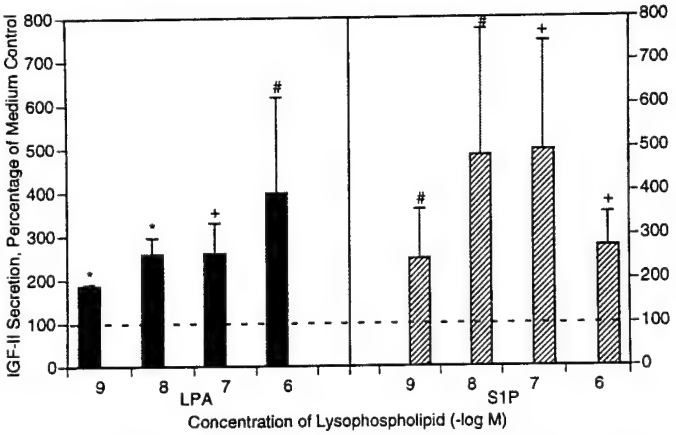

Fig. 5. Stimulation by LPA and S1P of MCF-7 BCC secretion of IGF-II. *Columns*, means of the results of three studies; *bars*, SD. Secretion of IGF-II in medium alone was 1.7, 3.0, and 4.1 ng/ml in the three studies. The statistical methods and symbols are the same as in Fig. 4, except that # = $P = 0.05$.

were detected in MDA-MB-453 BCCs, where the respective mean maxima were 24-fold and 26-fold.

Pharmacological inhibitors known to suppress one or more components of the pathways by which Edg Rs signal nuclear events were applied in BCCs transfected with the SRE-luciferase reporter. Suppression of Gi protein activity by PTX, the ras-mitogen-activated protein kinase pathway by a MEK inhibitor, and the rho pathway by C3 exoenzyme all substantially decreased nuclear signals from Edg receptors in both types of BCCs (Table 2).

Enhancement of BCC Secretion of IGF-II by LPA and S1P. Functional prominence of the IGF-II/IGFR1 system in many breast cancers suggested the possibility that part of the stimulation of proliferation of some lines of BCCs by LPA and/or S1P might be attributable to augmentation of secretion of IGF-II by one or both lysophospholipid mediators. Secretion of radioimmunoreactive IGF-II by MCF-7 BCCs was enhanced significantly by 10^{-9} M to 10^{-6} M LPA and S1P in concentration-dependent relationships where the maximal effects were attained by 10^{-6} M LPA and 10^{-8} M and 10^{-7} M S1P (Fig. 5). In two of the studies, neither 10^{-10} M LPA nor S1P affected release of IGF-II. At 10^{-6} M, but not 10^{-8} M, the phosphatidic acid and sphingosine biochemical precursors of LPA and S1P enhanced secretion of IGF-II with marginal statistical significance. A dot-blot immunoassay for IGF-II, which eliminates the blocking activity of IGF-binding proteins, gave similar results for MCF-7 BCCs. With 10^{-7} M LPA, 10^{-6} M LPA, 10^{-8} M S1P, and 10^{-7} M S1P, MCF-7 BCC-derived IGF-II was increased to respective means of 2.6-, 3.2-, 4.7-, and 5.5-fold above a mean unstimulated level of 2.2 ng/ml. Stimulation of MCF-7 BCC secretion of IGF-II by LPA and S1P was inhibited by PTX, MEK inhibition, and C3 exoenzyme sufficiently to implicate Gi and both the ras and rho pathways of signaling by the Edg receptors (Table 3). A greater involvement of signaling through the ras-raf-mitogen-activated protein kinase pathway than rho pathways may be predicted based on the higher effectiveness of the MEK inhibitor than C3 exoenzyme.

The level of secretion of IGF-II by LPA- and S1P-stimulated MDA-MB-453 BCCs was much lower than that by MCF-7 cells, and it was not possible to quantify accurately the very low IGF-II concentrations attained by unstimulated MDA-MB-453 cells. With 10^{-6} M LPA and 10^{-7} M S1P, the levels of IGF-II secreted by MDA-MB-453 BCCs attained means of 1.2 and 2.0 ng/ml, respectively. Because stimulated levels of IGF-II from MDA-MB-453 BCCs were only one-fifth of those from MCF-7 BCCs or lower and unstimulated levels were not reliably detectable, subsequent studies focused only on IGF-II mechanisms in MCF-7 BCCs. The capacity of human synthetic IGF-II to stimulate BCC proliferation, at concentrations in the range attained by incubation of MCF-7 BCCs with LPA and S1P, was examined to assess functional relevance of the observed endogenous increases. IGF-II increased MCF-7 BCC proliferation significantly, as determined by increases in cell counts after 72 h. MCF-7 BCC counts were increased by 1, 3, 10, and 30 ng/ml of IGF-II to respective means of 152, 234, 316, and 388% ($n = 2$) of serum-free medium control. The same range of concentrations of synthetic IGF-II also activated SRE in MCF-7 BCCs, as detected in the reporter assay (Table 4). The increases in SRE signal above control level were significant for all concentrations of IGF-II examined, and the increment in SRE signal attained by each higher concentration compared with the next lower concentration also was significant. The reduction in LPA-induced SRE signal by immunoneutralization of IGF-II was similar in magnitude to the maximum increase elicited by IGF-II alone (Table 4).

Suppression of MCF-7 BCC Responses to LPA and S1P by Anti-IGF-II and Anti-IGFR1 Antibodies. MCF-7 BCCs were preincubated with a range of concentrations of an IgG1 mouse neutralizing monoclonal anti-IGF II antibody, prior to introduction of 10^{-7} M LPA and S1P. The neutralizing antibody to IGF-II suppressed significantly both proliferative responses and SRE-luciferase reporter responses with antibody concentration dependence, whereas isotype-matched control IgG1 had no effect (Fig. 6). The effects of anti-IGFR1 antibody, which blocks binding of IGF-II to IGFR1, were examined in relation to the stimulatory effects of 10^{-7} M S1P on MCF-7 BCCs. At 1, 3, and 10 μg/ml, anti-IGFR1 antibody suppressed S1P-stimulated proliferation of MCF-7 BCCs, as assessed with cell counts, by means ± SD ($n = 3$) of 20 ± 4.6%, 32 ± 4.0%, and 41 ± 3.6% ($P < 0.01$ for all), respectively. At 3, 10, and 30 μg/ml, anti-IGFR1 antibody suppressed S1P-stimulated activation of the SRE-luciferase reporter in MCF-7 BCCs by means ± SD ($n = 3$) of 36 ± 7.8%, 47 ± 7.8%, and 51 ± 7.6% ($P < 0.01$ for all), respectively. In contrast, the IgG isotype control had no significant inhibitory effect, and anti-IGFR1 antibody did not suppress unstimulated proliferation of MCF-7 BCCs.

Table 3 *Pharmacological inhibition of LPA and S1P enhancement of MCF-7 BCC secretion of IGF-II*

Each value is the mean ± SD of the results of three studies. The significance of each level of inhibition was calculated by a paired Student *t* test. The levels of IGF-II in medium without an inhibitor were 7.4, 10, and 12 ng/ml for 10^{-7} M LPA and 6.1, 8.5, and 10 ng/ml for 10^{-7} M S1P.

	Lysophospholipid Signaling Inhibitor		
	PTX	MEK INH (mean inhibition ± SD)	C3 exoenzyme
LPA (10^{-7} M)	83 ± 14[a]	44 ± 8.3[a]	19 ± 15
S1P (10^{-7} M)	60 ± 19[b]	35 ± 3[a]	19 ± 2[b]

[a] $P < 0.01$.
[b] $P < 0.05$.

Table 4 *Activation of SRE-Luciferase reporter in MCF-7 BCCs by IGF-II*

Each value is the mean ± SD of the results of three studies. The significance of each level of stimulation relative to serum-free control without IGF-II or LPA (100%) was calculated by a paired Student *t* test. The levels of significance of differences between 1 and 3 ng/ml ($P < 0.01$), 3 and 10 ng/ml ($P < 0.05$), and 10 and 30 ng/ml ($P < 0.05$) of IGF-II and between LPA without and with anti-IGF-II neutralizing antibody ($P < 0.01$) were calculated by the same method.

IGF-II (ng/ml)					
1	3	10	30	LPA (10^{-7} M)	LPA (10^{-7} M) + anti-IGF-II (30 μg/ml)
178 ± 17[a]	209 ± 24[a]	260 ± 44[b]	316 ± 31[a]	1202 ± 152[a]	910 ± 165[a]

[a] $P < 0.01$.
[b] $P < 0.05$.

DISCUSSION

IGF-I and IGF-II potently stimulate proliferation of many types of normal and malignant cells (19, 20). The IGFR1 is a heterotetrameric complex with tyrosine kinase activity that binds and transduces signals from IGF-I and IGF-II similarly (21). IGFR2 differs structurally from IGFR1, lacks signal transduction functions, and does not mediate cellular proliferation (22). IGF-II is the predominant form in human cultured BCCs, stimulates BCC proliferation through IGFR1, and decreases the estrogen growth requirement of ER-positive BCCs (23). Estrogen is a potent stimulus of proliferation of ER-positive BCCs that concurrently enhances expression and secretion of IGF-II by such lines of BCCs (19). However, the possibility that the IGF system may not have a major role in estrogen enhancement of proliferation of some ER-positive BCCs was suggested by the lack of inhibition of estrogen stimulation when IGFR1 was blocked by a neutralizing monoclonal antibody (24). In contrast, stimulation of proliferation of BCCs by the lysolipid phosphate growth factors LPA and S1P appears to be mediated in part by IGF-II but is not dependent on the expression of ERs.

The ER-positive MCF-7 cells and ER-negative MDA-MB-453 cells both express Edg-2 and Edg-4 Rs for LPA and Edg-3 and Edg-5 Rs, but not Edg-1 Rs, for S1P, with quantitative differences in the respective levels (Figs. 1 and 2; Table 1). Significant ligand concentration-dependent stimulation of BCC proliferation by LPA and S1P was observed with both lines, irrespective of ER status (Fig. 3). Signaling of transcription of growth-related genes, as assessed by prominent enhancement of SRE-coupled luciferase activity, was increased significantly by proliferation-stimulating concentrations of LPA and S1P in both MCF-7 and MDA-MB-453 BCCs (Fig. 4). The suppression of SRE-coupled reporter responses to LPA and S1P by PTX and by inhibition of MEK and rho, in a pattern characteristic of signal transduction by Edg Rs, confirms the presence of functional Edg Rs in both BCC lines (Table 2).

LPA and S1P both significantly enhanced secretion of immunoreactive IGF-II by MCF-7 cells up to respective peaks 4- and 5-fold higher than control levels (Fig. 5). IGF-II secretion evoked by 10^{-7} M LPA or S1P was suppressed significantly by PTX and MEK inhibition and less significantly by C3 exoenzyme inactivation of rho, which also is consistent with Edg R mediation (Table 3). The role of IGF-II was explored first by investigating the stimulation of proliferation and SRE-luciferase activity in MCF-7 BCCs by a range of concentrations of purified synthetic IGF-II (Table 4). At concentrations elicited by LPA or S1P, the synthetic IGF-II evoked greater proliferation and SRE-luciferase activity than at concentrations attained by unstimulated MCF-7 BCCs. The role of native IGF-II was confirmed by defining the effects of neutralizing antibodies to IGF-II and IGFR1 on growth and SRE-reporter responses to 10^{-7} M LPA and S1P (Fig. 6). Both responses of MCF-7 cells were inhibited by means of up to 55 and 65%, respectively, without an effect of non-antibody isotype-identical IgG (Fig. 6). Thus, a substantial part of the stimulation of growth of some BCCs by LPA and S1P depends on increased release of IGF-II and its capacity to induce BCC proliferation.

A tentative integration of the present findings suggests distinctive functions for lysolipid phosphate mediators in BCC biology. At concentrations usually attained in serum and in some inflammatory and malignant exudates and plasma (1, 25, 26), LPA and S1P both exert dual effects on BCC proliferation. First, the SRE-luciferase responses not inhibited by anti-IGF-II or anti-IGFR1 neutralizing antibodies represent either direct nuclear signaling through Edg Rs or possibly the actions of other non-IGF protein growth factors elicited by the lysolipid phosphate mediators and capable of activating SRE. Second, LPA and S1P enhance generation and/or release of IGF-II by the

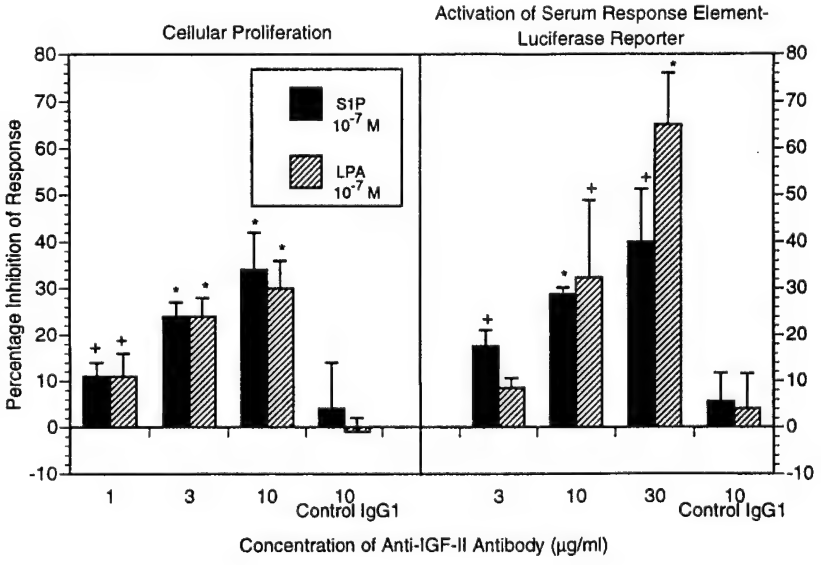

Fig. 6. Suppression of MCF-7 BCC responses to LPA and S1P by a neutralizing anti-IGF-II mouse monoclonal antibody. *Columns*, means of the results of three studies performed in duplicate; bars, SD. The control (0% inhibition) responses to 10^{-7} M LPA and S1P are shown in Figs. 3 and 4. The statistical methods and symbols are the same as in Fig. 4.

BCCs, irrespective of ER expression. The results of preliminary analyses of LPA and S1P production by BCCs showed very low endogenous levels, which would not have functional relevance. The sources of LPA and S1P, therefore, are likely to be cells other than the target BCCs, and these lysolipid phosphate growth factors thus would not appear to be autocrine stimuli in breast cancer. Rather, this class of mediators may function both as paracrine growth factors and by setting thresholds for secretory responses of one or more autocrine protein growth factors.

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Mechanisms of Lysolipid Phosphate Effects on Cellular Survival and Proliferation

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ABSTRACT: **The specificity of cellular effects of lysolipid phosphate (LLP) growth factors is determined by binding to endothelial differentiation gene–encoded G protein–coupled receptors (EDG Rs), which transduce diverse proliferative and effector signals. The primary determinants of cellular responses to LLPs are the generative and biodegradative events, which establish steady-state concentrations of each LLP at cell surfaces, and the relative frequency of expression of each EDG R. There are major differences among types of cells in the net effective generation of the LLPs, lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), and in their profile of expression of EDG Rs. The less well characterized secondary determinants of cellular specificity of LLPs are high-affinity binding proteins with carrier and cell-presentation functions, cell-selective regulators of expression of EDG Rs, and cellular factors that govern coupling of EDG Rs to G protein transductional pathways. The roles of components of the LLP-EDG R system in normal physiology and disease processes will be definitively elucidated only after development of animal models with biologically meaningful alterations in genes encoding EDG Rs and the discovery of potent and selective pharmacological probes.**

GENERATION, TRANSPORT, CELLULAR PRESENTATION, AND ACTIONS OF LYSOLIPID PHOSPHATE MEDIATORS

Primary and Secondary Mechanisms for Specificity

Lysophosphatidic acid (LPA), sphingosine 1-phosphate (S1P), and other structurally related lysolipid phosphates (LLPs) have major effects on diverse cellular func-

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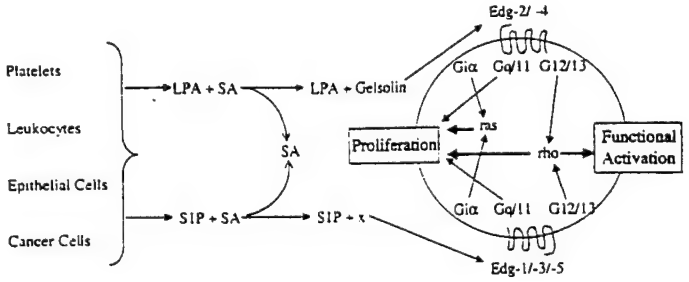

FIGURE 1. Generation, transport, and effects of lysolipid phosphate mediators. SA = serum albumin: x = postulated high-affinity S1P-binding protein.

tions, including initiation and regulation of proliferation, enhancement of survival, suppression of apoptosis, promotion of differentiation, and stimulation of cytoskeletal filament–based functions of many types of cells[1–4] (FIG. 1). LLPs are generated from precursors stored in membranes and secreted by platelets, macrophages, epithelial cells, and some cancer cells in amounts sufficient to establish micromolar concentrations in plasma normally and in other extracellular fluids during tissue reactions. LPA and S1P are both almost entirely bound by proteins in biological fluids. Serum albumin is a low-affinity and high-capacity carrier for LPA and S1P, whereas the actin-cleaving protein gelsolin is a high-affinity and low-capacity carrier for LPA, but not S1P.[5] It is presumed that S1P is bound by other, as yet unidentified, high-affinity proteins (FIG. 1). The present hypothesis is that LPA is bound by serum albumin and gelsolin in plasma and some other normal extracellular fluids, but almost exclusively by gelsolin at the surface of myocytes and other gelsolin-producing cells, where gelsolin exerts a predominant role in affinity-linked cellular presentation of LPA to EDG-2 and -4 Rs.[5] Normal plasma concentrations of gelsolin bind LPA with sufficient avidity to prevent optimal interactions with cellular receptors. In contrast, at the concentrations of approximately 5% to 15% of that of normal plasma found in many reactive extracellular fluids, gelsolin presents LPA to some types of cells with greater effectiveness than serum albumin.

LLPs resemble polypeptide growth factors (PGFs) in their capacity to evoke many cellular responses other than proliferation, act as autocrine and paracrine mediators, and signal cells through receptor-coupled transductional pathways, which alter transcriptional activities of growth-related genes directly and by amplification mechanisms.[3,4] Major differences between LLPs and PGFs are the cell membrane phospholipid precursor sources of LLPs, as contrasted with *de novo* synthesis of PGFs; multiple phospholipase- and phosphohydrolase-dependent enzymatic pathways for biodegradation of LLPs, as distinguished from proteolysis of PGFs; and their respective uses of G protein–coupled receptors (GPCRs) and protein tyrosine kinase receptors.

The central problem of biological specificity of the omnific LLPs, in contrast to PGFs, derives from the capacity of so many types of cells to produce and respond to LLPs. The primary determinants of specificity are the generative and biodegradative events, which establish steady-state concentrations of each LLP at cell surfaces, and

the structures, signaling pathways, and prevalence of each LLP receptor, which determine the net binding of LLPs and the characteristics of transduction. The secondary determinants of specificity are high-affinity carrier proteins and cell-selective presentation mechanisms, regulators of LLP receptor expression and signaling, and many other concurrently expressed mediator systems that modify cellular responses to LLPs. Thus, the next critical research goals for increasing our understanding of the distinctive roles of LLPs in normal physiology and disease processes are (1) to identify cell-selective factors that alter production, secretion, and biodegradation of each LLP, (2) to characterize the sources, nature, and cell-selective functions of LLP-binding proteins responsible for LLP transport in blood and other fluids and tissues, and for delivery to cellular receptors, (3) to delineate the distribution of LLP receptors on cells within each major organ system normally and in disease states, (4) to determine which factors regulate expression and signaling properties of LLP cellular receptors normally and in disease states, and (5) to define the major interactions between LLPs, PGFs, and other mediators of cellular functions.

In a few instances, it already has been demonstrated tentatively that an LLP is generated at greater than normal rates or that expression of one LLP receptor is expressed at higher than usual levels in relation to a developmental event, normal cellular response, or pathological process. Similarly, it has been shown experimentally in several model systems that LLPs may alter cellular production of PGFs or responsiveness to PGFs, and thereby increase the target cell selectivity of action of the LLPs. More conclusive correlation of these alterations in activity of the LLP mediator system with physiological and pathological events *in vivo* will require the availability of potent and selective pharmacological agents, functionally active antibodies to LLP receptors, and animal models with genetically overexpressed or deleted LLP receptors.

CELLULAR PATTERNS OF EXPRESSION OF EDG RECEPTORS FOR LLPs

Two subfamilies of G protein–coupled receptors (GPCRs), which are encoded by endothelial differentiation genes (*edg*s) and thus are designated EDG Rs, are dedicated to LPA, S1P, and related LLP mediators[6–11] (FIG. 1). The EDG Rs discovered so far may be considered in two homology and functional clusters based on both amino acid sequence identity and principal LLP ligand.[3,4] The first encompasses EDG-1, -3, -5, and -8, which are 45–60% amino acid sequence identical and bind S1P with high specificity.[6,9] The second includes EDG-2, -4, and -7, which exhibit 40–50% amino acid sequence identity and bind LPA with high affinity, but not S1P or other sphingolipids.[7,8,10] The EDG Rs all couple to three or more types of G proteins and transduce decreases in $[cAMP]_i$ through Gi, increases in $[Ca^{2+}]_i$ by augmenting phospholipase C activity through Gq/11 and beta/gamma dimers, and induction of PI_3 kinase, p125 focal adhesion kinase (FAK), and phospholipase D by activating rho through G12/13.[3,4] Induction of activity of serum response element (SRE) and subsequent transcriptional events by EDG Rs requires recruitment both of ternary complex factor (TCF) through Gi and ras, or through Gq/11 and the mitogen-activated protein (MAP) kinases ERK 1 and 2, and of serum response factor

(SRF) through G12/13 and rho.[12] All EDG Rs analyzed to date signal both nuclear transcriptional events and increases in $[Ca^{++}]_i$.[13–15] For each EDG R, however, a different G protein or combination of G proteins may serve as the predominant link to any one biochemical pathway.[16,17]

Assessment of mRNAs encoding EDG Rs by Northern blot and semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) techniques and of EDG R proteins by Western blots has provided preliminary, but often distinctive, profiles of their expression in several human and rodent organ systems. EDG-1 Rs dominate in endothelial cells and are present in lower amounts in some normal and anaplastic epithelial, neural, and myocytic cells, but are not detected in any cells of the T lymphocyte lineage from thymocytes to mature T helper and T suppressor cells. The EDG-2 R appears in dividing neurons of the periventricular zone in the developing murine brain and then disappears from postmitotic neurons of the adult brain.[7] In adult rodent and human brain cells, EDG-2 R is expressed only by oligodendrocytes.[18] EDG-4 R is absent at the level of protein and mRNA from freshly isolated normal human ovarian epithelial cells and SV40 virus–immortalized cultured lines of ovarian epithelial cells, but is expressed at high levels in all human ovarian cancer cell lines and tissues examined to date.[19,20] Rat cardiac myocytes express all EDG Rs except EDG-1, and the levels are increased by hypoxia and adrenergic agonists.[5] LPA acts through upregulated EDG-2 and EDG-4 Rs to protect cardiomyocytes from apoptosis induced by hypoxia and/or adrenergic stimulation. For the immune system, EDG-4 LPA R and EDG-6 R, for which the LLP ligand has not been identified as yet,[21] are the most densely represented in T cells, but also EDG-2, -3, and -4 are detected in some lines of human malignant T cells.[22,23] EDG-3 and -5 Rs are widely expressed in epithelial cells and fibroblasts. The results of studies to date indicate that levels of EDG Rs in thymocytes and T cells are altered substantially and differentially by cellular activation and some apoptosis-inducing agents. For example, ceramides, which enter T cells and evoke apoptosis, downregulate EDG-2 and EDG-4 LPA receptors, but not any of the S1P-specific EDG-Rs.[22] With the exception of the EDG-1 R,[6] however, very few examples of exclusive involvement of one type of EDG R and its signaling pathway have been delineated in relation to target cell specificity of LPA or S1P.

EFFECTS OF LLPs ON CELLULAR SURVIVAL AND PROLIFERATION

Regulation of T Lymphocyte Susceptibility to Apoptosis and Expression of Autocrine Polypeptide Growth Factors

LLPs affect cellular proliferation by four, often interactive, mechanisms. The first is enhancement of serum response element (SRE) activity in promoters of immediate-early growth–related genes.[12] The second is induction of cellular production and secretion of one or more polypeptide growth factors.[24,25] The third is sensitization of some types of cells to the effects of a polypeptide growth factor. This mechanism has been observed in cells for which LLPs alone have only weak activity, such as mesangial cells.[26] The fourth and rarest mechanism is inhibition of proliferation, as has been observed for some myelocytes in which LPA increases the intracellular

concentration of cyclic AMP ($[cAMP]_i$).[27] The results of recent studies of the roles of LLPs in cellular survival and proliferation often have revealed alterations in the cellular concentration, localization, or activity of one or two functionally relevant proteins of the target cells, which encompass diverse growth factors, receptors for growth factors, and other growth-related control proteins. Some of these mechanisms are well illustrated by the findings of investigations of T cell responses to LPA and S1P.

In the initial studies, LPA and S1P had striking effects on T cell susceptibility to apoptosis due to alterations in cellular levels of proteins of the Bcl-2 family and of the caspase cluster.[22,28] LPA and S1P also increased T cell sensitivity to diphtheria toxin (DT) as a result of enhanced T cell expression of the receptor for diphtheria toxin, which is heparin-binding epidermal growth factor–like growth factor (HB-EGF).[23] Cultured Tsup-1 cells of a human $CD4^+8^+3^{low}$ lymphoblastoma line express EDG-2, -3, -4, and -5 Rs, but not EDG-1 R, as determined by both RT-PCR analyses and Western blots.[22,23] Tsup-1 cell apoptosis was induced by antibodies to CD2, CD3 plus CD28 in combination, and Fas and by cell-permeant ceramide, and was assessed by morphological characteristics, increases in end-labeling of free 3′-OH groups of DNA, and release of radioactively labeled fragments of DNA. At 10^{-10} M to 10^{-7} M, both LPA and S1P protected Tsup-1 cells from apoptosis evoked by antibodies to surface proteins.[22] In contrast, S1P, but not LPA, suppressed apoptosis elicited by C6-ceramide. The failure of LPA to prevent ceramide-induced apoptosis of Tsup-1 cells was partially due to suppression by ceramide of the expression of EDG-2 and -4 Rs, but not EDG-3 and -5 Rs.[22] At 10^{-9} M to 10^{-7} M, both LPA and S1P suppressed Tsup-1 cell content of the apoptosis-promoting protein Bax without altering levels of Bcl-2 or Bcl-x_L.

The LPA and S1P suppression of Bax mediated by EDG Rs was shown by selectively reducing expression of EDG-2 and -4 together and of EDG-3 and -5 together through transfection of Tsup-1 cells with pools of the respective antisense cDNAs in plasmids expressing hygromycin resistance to allow enrichment of transfectants. Levels of suppression of EDG-2 and EDG-4 Rs that inhibited reductions in Bax by LPA prevented LPA protection from apoptosis.[22] Similarly, suppression of EDG-3 and -5 that inhibited reductions in Bax by the lower concentrations of S1P prevented S1P protection from apoptosis. At levels of S1P $\geq 10^{-7}$ M, prevention of Tsup-1 cell apoptosis correlated best with inhibition of activity of caspases 3, 6, and 7, but levels of LPA $> 10^{-7}$ M did not inhibit caspase activities in Tsup-1 cells or prevent apoptosis.

Other investigations of the effects of LPA and S1P on T cell survival revealed striking sensitization of Tsup-1 cells to the action of diphtheria toxin (DT). After 4 h of exposure of Tsup-1 cells to 1–10 ng/mL of DT, protein synthesis was suppressed by 11% to 72% and the levels of suppression were increased significantly by 10^{-9} M to 10^{-6} M LPA or S1P.[23] Heparin-binding epidermal growth factor–like growth factor (HB-EGF) is a plasma membrane protein of T cells, which binds to EGF Rs and matrix proteoglycans, and is the cellular receptor for DT. Under conditions that enhanced sensitivity to DT, LPA and S1P increased Tsup-1 cell expression of HB-EGF, as assessed by Western blots.[23] Direct evidence for the involvement of increased levels of HB-EGF in LLP enhancement of Tsup-1 cell sensitivity to DT was provided by HB-EGF neutralizing antibodies, which blocked the DT-sensitizing activity of

LPA and S1P. None of a range of analogues of LPA and S1P or other phospholipids mimicked the effects of the parent LLPs, and specific inhibitors of pathways of signaling characteristic of EDG Rs reduced LPA and S1P stimulation of both expression of HB-EGF and increased sensitivity to DT.

To confirm the roles of EDG Rs, Tsup-1 cells were transfected with EDG-2 plus EDG-4 antisense cDNA in mammalian expression plasmids encoding hygromycin resistance and incubated with hygromycin to augment the percentage of Tsup-1 cells with antisense suppression of EDG-2 and -4, as reflected in Western blots. Antisense reduction of EDG-2 and -4, but not EDG-3 and -5, prevented both increases in HB-EGF and enhanced sensitivity to DT induced by LPA, but not S1P.[23] Transfection of Tsup-1 cells with EDG-3 plus -5 antisense plasmids in the same protocol, to suppress immunodetectable EDG-3 and -5 proteins, prevented increases in both HB-EGF expression and sensitivity to DT elicited by S1P, but not LPA. In the absence of DT, such increased expression of HB-EGF may amplify LPA and S1P stimulation of T cell proliferation through greater juxtacrine activation of endogenous EGF Rs and heightened interactions of T cells with matrix proteoglycans. In preliminary studies of two lines of Jurkat human T cell transfectants stably overexpressing both EDG-3 Rs and EDG-4 Rs, 10^{-10} M to 10^{-7} M S1P and LPA respectively increased Jurkat T cell proliferation by up to 6-fold, as assessed by increased uptake of ^{3}H-thymidine. In wells precoated with heparan sulfate, the proliferation-enhancing effects of both LPA and S1P were increased further by a mean maximum of 3-fold. This effect is presumed to be attributable to increased expression of HB-EGF since neutralizing anti-HB-EGF antibody eliminated the stimulatory effect of heparan sulfate.

EFFECTS OF LLPs ON HUMAN BREAST CANCER CELLS

Cultured lines of estrogen receptor–positive (ER+) and ER– human breast cancer cells (BCCs) express EDG-2, -3, -4, and -5 Rs, without detectable EDG-1 R, as assessed by semiquantitative RT-PCR analyses and Western blots.[25] The rank order of prevalence in two lines of ER+ BCCs was EDG-3 ≥ -4 ≥ -5 ≫ -2 Rs and in two lines of ER– BCCs was EDG-3 > -4 ≫ -5 = -2 Rs (FIG. 2). Thus, both ER+ and ER– BCCs were predicted to respond to LPA and S1P. Detailed studies of the functional effects of LPA and S1P were conducted with the MCF-7 (ER+) and MDA-MB-453 (ER–) lines of human BCCs.[25] LPA and S1P at 10^{-8} M to 10^{-6} M enhanced proliferation of both BCC lines significantly after 72 h, as assessed by cell counts and ^{3}H-thymidine uptake, to maximal levels of 2.5- to 4-fold higher than that of control BCCs in serum-free medium alone. The level of SRE activity in BCCs transiently transfected with an SRE-luciferase reporter, which was used as an index of nuclear responses to proliferation-inducing LLP signals, was increased within 4 h by respective mean maxima of 37-fold and 85-fold by LPA and S1P in MCF-7 BCCs and by 24-fold and 26-fold in MDA-MB-453 BCCs.[25]

To examine the growth amplification mechanisms recruited by the LLPs (FIG. 2), their effects on secretion of the predominant type II insulin-like growth factor (IGF-II) were examined in MCF-7 BCCs that had readily quantifiable baseline levels not detected in culture media conditioned by the MDA-MB-453 BCCs. Significant in-

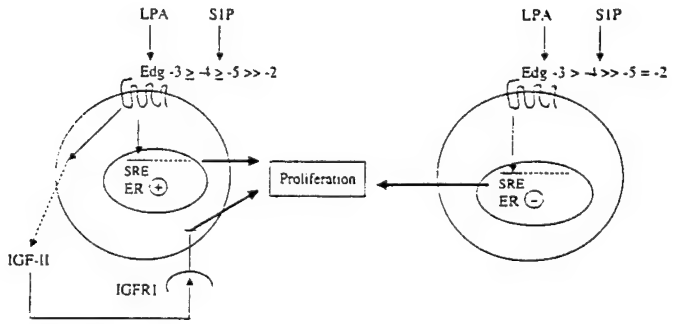

FIGURE 2. Lysolipid phosphate effects on human breast cancer cells. ER = estrogen receptor; SRE = serum response element; IGF-II = type II insulin-like growth factor; IGFR1 = type 1 insulin-like growth factor receptor.

creases in secretion of IGF-II by MCF-7 BCCs were evoked by 10^{-9} M to 10^{-6} M LPA and S1P, to respective mean maxima of 3.2-fold and 5.5-fold higher than the unstimulated mean of 2.2 ng/mL.[25] To affirm the functional significance of increases of this magnitude, the SRE-luciferase and proliferative responses of MCF-7 BCCs to synthetic IGF-II were examined in the range of increases elicited by LLPs. Concentration-dependent increases in proliferation were observed in response to 3 to 30 ng/mL of IGF-II, up to a mean maximal increase of 388% with 30 ng/ml of IGF-II. Similar increases in SRE-luciferase activity were evoked by the same concentrations of IGF-II, up to a mean maximal increase of 316% with 30 ng/mL of IGF-II. Known pharmacological inhibitors of EDG R signaling suppressed significantly and to the same extent LPA and S1P enhancement of BCC proliferation and IGF-II secretion.[25] The capacity of neutralizing monoclonal anti-IGF-II antibody to decrease BCC proliferative and SRE-luciferase responses to LLPs by up to 33% and 65%, respectively, confirmed the functional importance of the amplifying contribution of IGF-II recruited by LLPs. Thus, LLPs augment growth of BCCs through multiple EDG Rs by the dual mechanisms of direct nuclear signaling and stimulation of secretion of relevant quantities of IGF-II and perhaps other PGFs (FIG. 2).

EFFECTS OF LLPs ON HUMAN OVARIAN CANCER CELLS

As high levels of LPA in plasma and ascitic fluid of patients with ovarian cancer correlate with a poor prognosis, it was considered important to investigate the expression and functions of EDG Rs in human ovarian cancer cells (OCCs) as compared to nonmalignant ovarian surface epithelial cells (OSE). Analyses of mRNA encoding EDG Rs by semiquantitative RT-PCR showed that EDG-2 and -4 were the predominant Rs (FIG. 3). The most distinctive finding was of high levels of EDG-4 R mRNA in numerous established lines of OCCs, but not in SV40-immortalized nonmalignant OSE (IOSE) or normal human OSE.[19,20] In contrast, the level of EDG-2 R mRNA in IOSE and OSE cells was equal to or greater than that in OCCs, and both

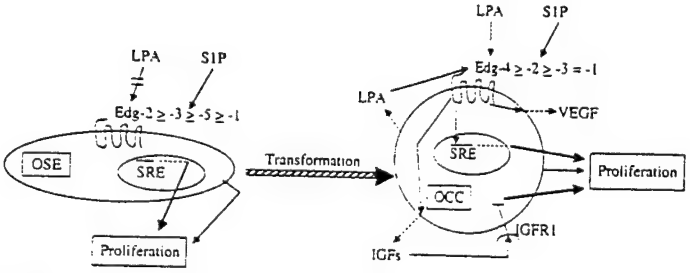

FIGURE 3. Lysolipid phosphate effects on human ovarian cancer cells. OCC = ovarian cancer cell; OSE = normal ovarian surface epithelial cell; SRE = serum response element; IGFs = a mixture of types I and II insulin-like growth factors; IGFR1 = type 1 insulin-like growth factor receptor.

EDG-3 and -5 R mRNA were consistently higher in IOSE and OSE cells than in OCCs.[19] EDG-1 R was expressed at similarly low levels in all lines of ovarian cells. Western blots supported the findings of higher levels of EDG-4 R in OCCs than in nonmalignant ovarian epithelial cells and higher levels of EDG-2, -3, and -5 Rs in IOSE and OSE cells than in OCCs[19] (FIG. 3). Thus, it was expected that OCCs would be more responsive functionally to LPA, and likewise nonmalignant ovarian cells to S1P.

LPA stimulated proliferation of the OV202 primary line of OCCs, but not IOSE 29 cells, as assessed by increases in uptake of ^{3}H-thymidine and cell counts[19] (FIG. 3). LPA evoked significant mean increases in uptake of ^{3}H-thymidine by OV202 cells of 1.7-fold at 10^{-6} M, 4.0-fold at 10^{-8} M, and 14-fold at 10^{-8} M, respectively, after 1, 3, and 5 days of stimulation. SRE-luciferase activity of OV202 OCC transfectants, which represents one index of immediate-early gene responses to EDG R signaling, was increased significantly by 10^{-9} M to 10^{-6} M LPA up to a mean maximum of 3-fold, whereas there was no response of IOSE 29 cell transfectants. In contrast, as predicted from the expression profile of EDG Rs, the SRE-luciferase responses to S1P were greater for IOSE 29 cells than OV202 cells.[19] OV202 OCC generation of IGF-II, which is a potent mitogen for OCCs, was increased significantly by 10^{-8} M and 10^{-7} M LPA and S1P to maximal levels of approximately 10-fold higher than medium alone. LPA also may promote ovarian tumor growth by increasing angiogenesis through stimulation of secretion of vascular endothelial growth factor (VEGF), which is the same protein as vascular permeability factor (VPF). LPA increased secretion of VEGF/VPF by the OVCAR-3 line of human OCCs up to a mean maximum of 4-fold, through a transcriptional activation mechanism, without influencing VEGF/VPF secretion by IOSE 29 cells[20] (FIG. 3). Pharmacological inhibitors of EDG R transduction suppressed similarly LLP stimulation of OCC proliferation, IGF-II generation, and VEGF production and secretion.[19,20] The capacity of some OCCs to secrete functionally relevant amounts of LPA suggests that the LLP-EDG R axis may be an autocrine growth and angiogenesis system in ovarian cancer (FIG. 3). The upregulation of VEGF/VPF also may contribute to the ascites, which is so characteristic of the local peritoneal invasion by ovarian cancer. EDG-4 R may be a marker for malignant transformation of ovarian

epithelial cells, as well as a transducer of proliferation by direct nuclear signaling and enhancement of secretion of IGFs and other PGFs.

SUMMARY AND RESEARCH PLANS

Cells in many organ systems produce LLPs and express EDG Rs in often distinctive and defining patterns. The signals transduced by EDG Rs, which stimulate cellular survival and proliferation, and evoke cellular functional responses, include direct nuclear messages, increases in the levels of endogenous mediators, enhancement of sensitivity to endogenous and exogenous factors, and amplification or reorientation of one or more of the basic signaling pathways. Biological specificity of the LLP-EDG R systems is regulated at many levels, but presently the roles of high-affinity transport and presentation proteins, the relative levels of expression of each EDG R, and cell-selective amplification mechanisms appear to be more important determinants than production and degradation of the LLPs. One clear exception is some malignancies, such as ovarian cancer, where the combination of production of large amounts of LPA and expression of high levels of EDG-2 and -4 Rs can create an autocrine growth system. In addition to conducting further studies of the basic characteristics of the LLP-EDG R system, it is critical to develop mouse models with genetically altered EDG Rs and appropriately specific and potent pharmacological agonists and antagonists for *in vivo* investigations.

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DUAL MECHANISMS FOR ENDOTHELIAL DIFFERENTIATION GENE-ENCODED RECEPTOR (EDG R) MEDIATION OF PROLIFERATION OF HUMAN BREAST CANCER CELLS (BCCS).

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The binding of sphingosine 1-phosphate (S1P) by G protein-coupled Edg-1, -3 and -5 Rs and of lysophosphatidic acid (LPA) by Edg-2 and -4 Rs initiates ras- and rho-dependent signaling of cellular growth and growth-related responses. MCF-7 and MDA-MB-453 cultured lines of BCCs express Edg Rs with a frequency of Edg-3>Edg-4>Edg-5> or =Edg-2, without detectable Edg-1, as determined by quantification of mRNA using reverse transcription-polymerase chain reaction and Western blots with anti-Edg R mouse monoclonal antibodies. Proliferation of both lines of BCCs, assessed by increased uptake of 3H-thymidine, was stimulated significantly up to four-fold by 10^{-9} M to 10^{-6} M LPA or S1P. Transfectional introduction into the BCCs of luciferase constructs containing the serum response element (SRE) of growth-related gene promoters demonstrated mean maximal SRE activation of 37- and 85-fold by LPA and S1P, respectively. Proliferative and SRE reporter responses of the BCCs to LPA and S1P were suppressed significantly by pertussis toxin (PTX), MEK inhibition and C3 exoenzyme. LPA and S1P stimulated MCF-7 cell secretion of type II insulin-like growth factor (IGF-II) by two- to seven-fold through a PTX-sensitive mechanism. A neutralizing monoclonal anti-IGF-II antibody suppressed proliferative and SRE reporter responses of BCCs to LPA and S1P by mean maxima of 50-54%. Thus Edg Rs mediate BCC growth responses to LPA and S1P both directly by activation of SRE and indirectly by enhancing the contribution of IGF-II. (Supported by NIH HL 31809)

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EFFECT OF 1,25-DIHYDROXYVITAMIN D_3 ON BREAST CANCER CELL GROWTH

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1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the biologically active form of vitamin D, inhibits proliferation of breast cancer cells and causes regression of rodent mammary tumors in vivo. The actions of $1,25(OH)_2D_3$ on cell growth and differentiation are mediated by nuclear receptor signals and rapid direct effects on cellular membranes.

Lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) are generated enzymatically from membrane lipids which stimulate proliferation, enhance survival and inhibit apoptosis of diverse types of cells. Members of a subfamily of G protein-coupled receptors encoded by endothelial differentiation genes (Edg Rs) are specific for either S1P (Edg-1,3,5 and 8) or LPA (Edg-2,4 and 7). LPA and S1P, at 10^{-8}-10^{-6}M, significantly stimulated the proliferation of MDA-MB-453 breast cancer cells (BCCs). To determine if $1,25(OH)_2D_3$ inhibits proliferation of BCCs by altering expression and transductional functions of Edg Rs, Edg Rs first were assessed by semi-quantitative RT-PCR and later by Real Time Quantitative PCR. MDA-MB-453 BCCs express predominantly Edg-3 > Edg-4 > Edg-5 > Edg-2 Rs, which transduce proliferative responses by nuclear signaling, through ras-MAP kinase and rho-dependent pathways, and by stimulating secretion of type II insulin-like growth factor.

The proliferative response of S1P-stimulated BCCs was suppressed extensively by 10^{-10}-10^{-8} M $1,25(OH)_2D_3$, as quantified by cell counts and Renilla luciferase-corrected reports of SRE-luciferase plasmids transfected into BCCs.

Elucidation of mechanisms for $1,25(OH)_2D_3$ effects on Edg R expression and signaling are expected to further our understanding of abnormal growth regulatory mechanisms in breast cancer and may lead to Edg R-directed modalities of treatment.

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Suppression by 1,25-Dihydroxyvitamin D3 of Expression of Endothelial Differentiation Gene - Encoded G Protein - Coupled Receptors (EDG Rs) for Lysophospholipid Growth Factors.

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Endothelial differentiation gene-encoded G protein-coupled receptors (EDG Rs) transduce major effects of the lysophospholipid growth factors, lysophosphatidic acid (LPA) and sphingosine-1 phosphate (S1P). EDG-1,-3,-5, -6 and –8 are specific for S1P and EDG-2,-4, and -7 are specific for LPA. LPA and S1P potently stimulate proliferation, enhance survival and inhibit apoptosis of many different normal and malignant cells. As the mechanism by which 1,25- dihydroxyvitamin D3 (VD3) inhibits breast cancer cell (BCC) growth is not fully known yet, we investigated whether the effects of VD3 include inhibition of LPA- and S1P- enhancement of proliferation of BCCs by suppressing EDGR expression and signaling. MDA-MB-453 BCCs express EDG-3 > EDG-4> EDG-5> EDG-2 Rs without detectable EDG-1. VD3 suppressed significantly the levels of mRNA encoding EDG-2 (mean-65%), -3 (mean –50%), and –5 (mean-40%), but had a lesser effect on EDG-4 as quantified by TaqMan Real Time PCR technique. VD3 also inhibited fluorimetrically-determined Ca2+ mobilization induced by LPA and S1P stimulation of EDG Rs. VD3 inhibition of proliferative responses of LPL-stimulated BCCs was assessed directly with a proliferation assay and quantification of stimulation of serum response element (SRE) transcription activity with renilla luciferase-corrected reports from an SRE-luciferase plasmid transfected into the BCCs. The EDG R-selectivity of VD3 suppression suggests both a novel cellular mechanism and the potential for specific therapy of some human breast cancers.